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Investigation of the role of the mucosa-associated microflora in feline alimentary lymphoma

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Table of contents

Summary

Persistent bacterial infections are causally linked to gastrointestinal cancer in people, and the concept that mucosa-associated bacteria can promote carcinogenesis is well established. Perturbation of the intestinal microbiome has been documented in cats with IBD and it seems plausible it could serve as a trigger for intestinal lymphomagenesis. It is against this background that we sought to assess the presence and localization of mucosal bacteria in biopsies from cats with histologically minimally changed small intestine (MC), lympho-plasmacytic enteritis (LPE), small (SCL), and large cell (LCL) alimentary lymphoma. Cases were identified in the histopathology database at Cornell. The spatial distribution (luminal debris, mucus, adherent to epithelium and serosa, invasion, intravascular presence) and numbers of bacteria were determined by fluorescence in situ hybridization (FISH) with the eubacterial probe EUB-338. Invasive bacteria were most frequently associated with LCL (82%). Invasion was not restricted to superficial areas of eroded or ulcerated mucosa, with translocated bacteria visualized in vessels and/or the serosa of 30% LCL. These results reveal that alimentary lymphoma is associated with alterations in the number and spatial distribution of mucosa-associated bacteria. They support the need for further study to speciate lymphoma associated bacteria, and careful evaluation of cats with alimentary lymphoma for evidence of septicemia.

Zusammenfassung

Ein kausaler Zusammenhang zwischen persistierenden bakteriellen Infektionen und gastrointestinalen Neoplasien beim Menschen, sowie das Konzept, dass mukosa-assoziierte Bakterien die Karzinogenese fördern können, sind weit bekannt. Veränderungen in der intestinalen Mikroflora wurden bei Katzen mit IBD dokumentiert und es scheint möglich, dass diese an der Entstehung des alimentären Lymphoms beteiligt sind. Vor diesem Hintergrund haben wir das Vorhandensein und die Verteilung von Bakterien in minimal veränderten Dünndarmbiopsien (MC), lymphoplasmazytärer Enteritis (LPE), sowie klein- (SCL) und grosszelligem (LCL) alimentärem Lymphom untersucht. Fälle wurden in der Histologie Datenbank der Cornell University eruiert. Die räumliche Verteilung (luminaler Debris, Mukus, Adhärenz am Epithel oder der Serosa, Invasion, intravaskulär) sowie die Anzahl der Bakterien wurde mittels Fluoreszenz in situ Hybridisierung (FISH) und der eubakteriellen Sonde EUB-338 evaluiert. Invasive Bakterien waren am häufigsten mit LCL assoziiert (82%) und nicht beschränkt auf oberflächliche oder ulzerierte Bereiche. Intravaskulär und/ oder serosal translozierte Bakterien wurden in 30% der LCL gesehen. Diese Resultate zeigen, dass das alimentäre Lymphom einhergeht mit Veränderungen der intestinalen Mikroflora und dass weitere Studien nötig sind, um die beteiligten Bakterien besser zu charakterisieren. Patienten mit alimentärem Lymphom sollten ausserdem streng auf die Anzeichen einer Septikämie überwacht werden.

1. Introduction

Lymphomas represent up to ninety percent of hematopoietic tumors in the cat and overall are one of the most frequently diagnosed neoplasms of domestic cats (Richter, 2003; Vail, 2007; Wilson, 2008). Although lymphomas usually arise in lymphoid tissue, they can potentially affect any tissue and various anatomic forms of feline lymphoma that differ in incidence are known (Louwerens, London, Pedersen, & Lyons, 2005; Richter, 2003; Vail, 2007; Wilson 2008). During the feline leukemia virus (FeLV) era of the 1960s through the 1980s, FeLV was the most common cause of lymphoma with up to 70% of cases, primarily of the cranial mediastinal, multicentric, renal and central nervous form, being associated with FeLV antigenemia (Francis, Cotter, Hardy, & Essex, 1979; Louwerens et al., 2005; Vail, 2007; Wilson, 2008). Detection of FeLV antigen is less frequently associated with the gastrointestinal form and reportedly occurs in 0% to 38% of cases (Jackson, Haines, Meric, & Misra, 1993; Mahony, Moore, Cotter, Engler, Brown, & Penninck, 1995; Richter, 2003; Vail, Moore, Ogilvie, & Volk, 1998; Zwahlen, Lucroy, Kraegel, & Madewell, 1998). An indirect role in lymphomagenesis has also been attributed to the feline immunodeficiency virus (FIV) (Vail, 2007). The introduction of routine testing and elimination programs in the 1970s and commercially available vaccines in the early 1980s greatly reduced FeLV infection rates (Louwerens et al., 2005; Vail, 2007). However, contrary to expectations and despite a decline in FeLV-associated lymphoma, the overall prevalence of lymphoma in cats has been increasing in the post-FeLV era, accompanied by a change in patient signalment and frequency of affected anatomic sites (Louwerens et al., 2005, Vail, 2007). An increase in intestinal lymphoma is mainly responsible for the apparent trend, with alimentary lymphoma now considered the most common anatomic form (Louwerens et al., 2005, Mahony et al., 1995; Vail et al., 1998). In contrast to mediastinal or multicentric lymphoma that are still FeLV-associated in younger cats, alimentary lymphoma most often affects older cats that are FeLV-negative (ELISA) (Louwerens et al., 2005, Mahony et al., 1995; Vail et al., 1998).

Histopathologically, two main types of alimentary lymphoma, low grade (small cell, lymphocytic) and high grade (large cell, lymphoblastic) lymphoma, can be distinguished, that differ in clinical presentation and response to therapy (Kiselow, Rassnick, McDonough, Goldstein, Simpson, Weinkle, & Erb, 2008; Richter, 2007; Wilson, 2008). Cats suffering from low grade alimentary lymphoma tend to have a more long-standing history of weight loss or other gastrointestinal signs, such as vomiting, anorexia, and diarrhea and rarely present with acute symptoms attributable to intestinal obstruction or perforation (Fondacaro, Richter, Carpenter, Hart, Hill, & Fettman, 1999; Kiselow et al., 2008; Richter, 2003; Vail, 2007; Wilson, 2008). In many cases, the history is found to be non- specific, symptoms minimal, and the physical exam normal with the exception of potentially thickened intestinal loops or abdominal lymph nodes (Richter, 2003; Vail, 2007; Wilson, 2008). Patients presenting with a palpable intestinal mass or septic abdomen due to perforation or intussusception, that may or may not have a history of preceding gastrointestinal signs, are more likely to have the much faster progressing high grade lymphoma (Richter, 2003; Vail, 2007; Wilson, 2008). With a median survival time of 18 to 24 month, prognosis for cats with small cell alimentary lymphoma treated with Chlorambucil is comparatively good, whereas cases of large cell alimentary lymphoma require more aggressive treatment protocols and have a worse prognosis (Fondacaro et al., 1999; Kiselow et al., 2008; Vail, 2007; Wilson, 2008). For the sake of completeness, it should be mentioned, that an intermediate grade type lymphoma and a large, granular cell lymphoma can also be found in some grading systems for alimentary lymphoma, but that those are not as well characterized as the above mentioned two types (Richter, 2003; Wilson, 2008). It seems that large, granular cell lymphoma is a very aggressive form of lymphoma that seldom has a good response to

chemotherapy, whereas intermediate cell lymphoma tends to behave more aggressively than small cell lymphoma and requires to be treated accordingly, but is still slower in progression than large cell lymphoma (Richter, 2003; Wilson, 2008).

In the early post-FeLV era (1990s) the vast majority of alimentary lymphoma cases were high grade type and of B-cell origin, whereas most of the other types of lymphoma were of T-cell lineage (Mahony et al., 1995; Richter, 2003; Vail et al., 1998). This supported the conclusion that the large cell type and B-cell phenotype in alimentary lymphoma often correlate and is consistent with the assumption that FeLV was more likely to transform T-lymphocytes as seen in other forms of lymphoma (Jackson, Wood, Misra, & Haines, 1996; Rojko, Kociba, Abkowitz, Hamilton, Hardy, Ihle, & O'Brien, 1989; Wilson, 2008). However, positive PCR results for FeLV in up to 70% of feline alimentary lymphomas (33% in T-cell, and 71 % in B-cell intestinal lymphoma), undermined the segregation of alimentary lymphoma on the basis of FeLV association, regardless of their lineage (Jackson et al., 1996; Richter 2003; Wilson, 2008).Given the decline in FeLV, it seems highly unlikely that latent FeLV infection is driving the increase in alimentary T cell lymphoma, but this remains to be confirmed (Richter, 2003; Vail et al., 1998).

The histomorphology of human alimentary lymphoma broadly parallels the situation in cats, where two histomorphologically distinct types of enteropathy-type T-cell lymphoma (ETL) occur in people (Chott, Haedicke, Mosberger, Födinger, Winkler, Mannhalter, & Müller-Hermelink, 1998; DeLeeuw, Zettl, Klinker, Haralambieva, Trottier, Chari, Ge, Gascoyne, Chott, Müller-Hermelink, & Lam, 2007; Isaacson & Du, 2005; Jaffe, 2009; Ko, Karnan, Kim, Park, Kang, Kim, Kang, Kim, Kim, Lee, Chun, & Seto, 2010; Zettl, DeLeeuw, Haralambieva, & Müller-Hermelink, 2007). ETL is a rare, primary extranodal, non-Hodgkin T-cell lymphoma that is thought to arise from intraepithelial lymphocytes (IEL) (Chott et al., 1998; DeLeeuw et al., 2007; Ko et al., 2010; Van de Water, Cillessen, Visser, Verbeek, Meijer, & Mulder, 2010; Zettl et al., 2007). The proximal jejunum is the site affected by ETL most commonly, whereas involvement of other parts of the small intestine, the stomach or large intestine occurs less frequently (Delabie, Holte, Vose, Ullrich, Jaffe, Savage, Connors, Rimsza, Harris, Müller-Hermelink, Rüdiger, Coiffier, Gascoyne, Berger, Tobinai, Au, Liang, Montserrat, Hochberg, Pileri, Federico, Nathwani, Armitage, & Weisenburger, 2011; Isaacson & Du, 2005; Zettl et al., 2007). At time of presentation, up to 40% of patients suffer from an acute abdomen with peritonitis in consequence of intestinal perforation or obstruction, highlighting that the clinical course of ETL is highly aggressive (Delabie et al., 2011; Isaacson & Wright, 1978; Zettl et al., 2007).

Type 1 ETL is far more common (80- 90% of ETL) than Type 2 ETL (DeLeeuw et al., 2007; Van de Water et al., 2010). Type 1 ETL is composed of pleomorphic, anaplastic, or immunoblastic tumor cells that typically are of a CD3+, CD8-, and CD56- immunophenotype (Chott et al., 1998; Delabie et al., 2011; DeLeeuw et al., 2007; Isaacson & Du, 2005; Zettl et al., 2007). The mucosa adjacent to the tumor in cases of Type 1 ETL shows increased numbers of IELs, crypt hyperplasia, and villus atrophy in a majority of cases (Chott et al., 1998; DeLeeuw et al., 2007; Isaacson & Wright, 1978; Zettl et al., 2007). The less frequent Type 2 ETL is comprised of monomorphic, small to medium sized tumor cells with a predominantly CD3+, CD8+, and CD56+ immunophenotype (Chott et al., 1998; Delabie et al., 2011; DeLeeuw et al., 2007; Isaacson & Wright, 1978; Zettl et al., 2007). Evidence of an adjacent enteropathy is only present in about 50% of Type 2 cases (Chott et al., 1998; DeLeeuw et al., 2007).

ETL shows striking similarities to celiac disease (CD), such as the primary sites of gut involvement and the enteropathy features histologically seen in uninvolved tissue bordering the tumor (Delabie et al., 2011; Isaacson & Wright, 1978; Isaacson & Du, 2005; O'Farrelly, Feighery, O'Brian, Stevens, Connolly, McCarthy, & Weir, 1986; Zettl et al., 2007). Likewise, ETL is frequently associated with a

clinical history of short to long-standing malabsorption resembling CD (Chott et al., 1998; DeLeeuw et al., 2007; Isaacson & Wright, 1978; O'Farrelly et al., 1986; Van de Water et al., 2010; Zettl et al., 2007). Accounted for by the fact that histologic features of underlying enteropathy are found even in cases that do not have a clear clinical history of malabsorption, it was believed that both types of ETL are associated with CD for a long time (Chott et al., 1998; DeLeeuw et al., 2007; Isaacson & Wright, 1978; O'Farrelly et al., 1986; Van de Water et al., 2010; Zettl et al, 2007). More recent whole genome analyses and HLA genotyping of affected patients, however, confirmed that the histologically and immunohistochemically different Type 1 and Type 2 ETL are indeed two distinct lymphoma subtypes (DeLeeuw et al., 2007). While sharing the two genetic alterations gain of 9q and loss of 16q, Type 1 ETL shows gains of 1q and 5q significantly more often than Type 2 ETL, which by contrast is characterized by gains of the *MYC* oncogene locus (DeLeeuw et al., 2007). Furthermore, Type 1 ETL is associated with the HLA genotypes HLA-DQ2 and HLA-DQ8 that are essential for development of CD in 73% of cases, as compared to 33% in ETL Type 2, which corresponds to the incidence of this genotype in the normal Caucasian population (DeLeeuw et al., 2007). These findings suggest that only Type 1 ETL develops as a complication of CD and that at least one additional, different pathway is responsible for spontaneous neoplastic degeneration of IELs as seen in Type 2 ETL (DeLeeuw et al., 2007). As Type 2 ETL is morphologically very similar to feline small cell lymphoma, it seems crucial to define inflammatory triggers other than CD that can promote carcinogenesis.

Persistent bacterial infections are increasingly recognized as triggers for inflammation associated neoplastic transformation. The concept that mucosa-associated bacteria can promote gastrointestinal carcinogenesis was initially established through study of *Helicobacter pylori*. *H. pylori* infects 50% of the world's population and is known to play an important role in inducing chronic gastric inflammation and its progression to both, gastric carcinoma, as well as gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) (Fox & Wang, 2007; Mueller, O'Rourke, Chu, Chu, Dixon, Bouley, Lee, & Falkow, 2005). Representing the fourth most common cancer worldwide, gastric cancer has been extensively studied and the pathogenesis of gastric carcinoma is comparably well understood (Fox & Wang, 2007). Bacterial and host factors seem to be similarly responsible for the stepwise development of chronic inflammation following *Helicobacte*r persistence, mucosal atrophy, metaplasia, dysplasia, and lastly gastric carcinoma (Fox & Wang, 2007). Infection with an *H.pylori* strain able to express a type IV secretion system (T4SS) induces a proinflammatory environment in the gastric mucosa by delivering bacterial peptidoglycans and the cytotoxic protein CagA to epithelial cells (Fox & Wang, 2007). Recognition of the bacterial components leads to NF-kB activation and an up regulation of proinflammatory cytokines, such as IL-8 and IL-1 (Fox & Wang, 2007). In susceptible individuals chronic inflammation and hypergastrinemia promote mucosal atrophy, decreased secretion of gastric acid, an imbalance of proliferation and apoptosis in the chronically inflamed environment, and epithelial dysplasia (Fox & Wang, 2007). Achlorhydria associated proliferation of other bacteria species in the altered gastric environment may contribute to carcinoma development by producing nitrate reductase and enabling carcinogenic nitrosamine formation (Fox & Wang, 2007).

Although the human stomach is typically devoid of organized lymphoid tissue, it is the most common site affected by primary MALT lymphoma (Isaacson & Du, 2005). Chronic inflammation due to *H.pylori* infection can induce organized lymphoid follicles and subsequently progression to gastric MALT lymphoma (Isaacson & Du, 2005; Mueller et al., 2005). Histologically, the low-grade, B-cell type gastric MALT lymphoma still resembles the typical architecture of Peyer's patches (Isaacson & Du, 2005). Most neoplastic cells exhibit similarity to the small lymphocytes of the germinal center,

although a variable number of lymphoblasts can be intermixed with the well-differentiated cells (Isaacson & Du, 2005). High grade transformation of cells is less frequent and often results in a less favorable disease outcome (Mueller et al., 2005). The lymphoma diffusely infiltrates the region of the mucosa around the follicles, simulating the marginal zone of Peyer's patches (Isaacson & Du, 2005). The current hypothesis for the development of gastric MALT lymphoma illustrates the pathogenesis as a multistep process that starts out with *H.pylori* infection (Isaacson & Du, 2005). T-cells and B-cells, as well as other inflammatory cells are recruited to the gastric mucosa, where B-cell proliferation is stimulated mainly by *H.pylori* specific T-cells and partially also by auto-antigens (Isaacson & Du, 2005). In the context of constant stimulation one or more B-cell clones from the acquired MALT can undergo malignant transformation by acquiring chromosomal alterations (Isaacson & Du, 2005; Mueller et al., 2005). A number of genetic abnormalities, particularly three chromosomal translocations, can be found in gastric MALT lymphoma that are specifically associated with it: t(11;18)(q21;q21), t(1;14)(p22;q23), and t(14;18)(q32;q21) (Auer, Gascoyne, Connors, Cotter, Greiner, Sanger, & Horsman, 1997; Isaacson & Du, 2005; Streubel, Lamprecht, Dierlamm, Cerroni, Stolte, Ott, Raderer, & Chott, 2003). The transcription products of the altered genes at all three translocation loci are thought to be activators of the transcription factor NF-kB and in this way up regulate cytokines and growth factors that are important for cellular proliferation and survival (Isaacson & Du, 2005). It is generally accepted that *H.pylori* represents the antigenic stimulus that is necessary to activate the lympho-proliferative process proceeding neoplastic degeneration in gastric MALT lymphoma (Isaacson & Du, 2005; Mueller et al., 2005). The strongest evidence that *H.pylori* is not only associated with cases of gastric MALT lymphoma but causally linked to its development provides the fact that 75% of early stage tumors completely regress after eradication of the bacteria by use of antibiotic therapy (Isaacson & Du, 2005; Mueller et al., 2005). Only cases that reached the stage of chromosomal translocations or of advanced clinical stage seem to be *H.pylori* independent and unresponsive to antibiotic treatment (Isaacson & Du, 2005).

Just as in human gastric cancer, there is some evidence of association of *Helicobacter spp.* with feline gastritis and lymphoma (Bridgeford, Marini, Feng, Parry, Rickman, & Fox, 2008). As opposed to people, colonization with *Helicobacter spp*. in pet cats occurs in 91%, with *H. heilmannii (Hhe)* being the most prevalent species (Neiger, Dieterich, Burnens, Waldvogel, Corthésy-Theulaz, Halter, Lautenburg, & Schassmann, 1998). *H. heilmannii* strains *Hhe 2* and *Hhe 4* colonization was found to be associated with cats suffering from gastritis or gastric lymphoma, especially lymphoblastic lymphoma (Bridgeford et al., 2008; Priestnall, Wiinberg, Spohr, Neuhaus, Kuffer, Wiedmann, & Simpson, 2004). In contrast to human studies that succeeded to demonstrate a causative role of *Helicobacter spp.* in lymphoma development, further studies are needed to investigate the association of *H. heilmannii* with feline gastric lymphoma (Bridgeford et al., 2008; Fox & Wang, 2007).

Another inflammatory disease that may transform to a lymphoid neoplasia is immunoproliferative small intestinal disease (IPSID). IPSID is a rare immunoproliferative disorder in people that predominantly occurs in the Middle East and North Africa (Al-Saleem & Al-Mondhiry, 2005; Lecuit, Abachin, Martin, Poyart, Pochart, Suarez, Bengoufa, Feuillard, Lavergne, Gordon, Berche, Guillevin, & Lortholary, 2004). IPSID most commonly involves the proximal small bowel and is typified by dense infiltrates of centrocyte-like cells and plasma cells resembling low-grade B-cell MALT lymphoma (Al-Saleem & Al-Mondhiry, 2005; Isaacson, 1999; Lecuit et al., 2004; Parsonnet & Isaacson, 2004). A progression to high-grade, large-cell lymphoma is possible (Al-Saleem & Al-Mondhiry, 2005). Given its expression of a truncated immunoglobulin α heavy chain that is missing a V region and an

associated light chain, IPSID is also known as “alpha chain disease“(Al-Saleem & Al-Mondhiry, 2005; Lecuit et al., 2004; Parsonnet & Isaacson, 2004). Much like gastric MALT lymphoma, remission of early stage IPSID can be seen following antibiotic therapy, suggesting that a bacterial infection might be involved in carcinogenesis (Al-Saleem & Al-Mondhiry, 2005; Lecuit et al., 2004; Parsonnet & Isaacson, 2004). In 2004, Lecuit et al. were the first to show an association of *Campylobacter jejuni* and IPSID and suggest that it played the same role in the pathogenesis of IPSID as does *H. pylori* in in gastric MALT lymphoma (Lecuit et al., 2004; Parsonnet & Isaacson, 2004).

Perturbation of the intestinal microbiome in cats with idiopathic inflammatory bowel disease (IBD) (Janeczko, Atwater, Bogel, Greiter-Wilke, Gerold, Baumgart, Bender, McDonough, McDonough, Goldstein, & Simpson, 2008) is associated with patterns of mucosal cytokine upregulation and atrophy that are similar to those described in ETL and gastric cancer. These observations suggest that mucosal bacteria associated with IBD might serve as a trigger for the development of feline intestinal lymphoma of the B-, as well as the T-cell type.

It is against this background that we sought to determine the presence, number, and spatial distribution of mucosal bacteria in intestinal biopsies from cats with histologically normal small intestine, lympho-plasmacytic enteritis, and alimentary lymphoma.

2. Materials and Methods

2.1 Case selection

The database of the Section of Anatomic Pathology in the College of Veterinary Medicine, Cornell University (Ithaca, NY, USA) was searched in June 2011 for endoscopic and surgical feline intestinal biopsies and intestinal samples originating from necropsies that had been collected during the years 2007 through 2011. The search yielded a list of 551 in-house as well as referral cases and the histopathology reports were reviewed. A total of 353 case reports specifically characterized the small intestinal sections: normal or minimal change only (n=22), idiopathic inflammatory bowel disease (n=179), and alimentary lymphoma (n=152). 198 cases were not considered further because there were no sections available from the small intestine, specific causes of gastroenteritis could be demonstrated (e.g. bacterial overgrowth, parasite infestation, lesions resembling feline infectious peritonitis (FIP) or infection with FeLV, toxic, ischemic, or traumatic events), or neoplasias other than lymphoma were present (e.g. adenocarcinoma, leiomyosarcoma, hemangiosarcoma, mast cell tumor).

Sixteen cases interpreted as normal to minimally changed according to the original histopathology report were included in this study. Of the six cases rejected, five had intestinal sections harvested at necropsy and autolysis made assessment of the mucosa impossible and one tested positive for FIV. Endoscopic, surgical and necropsy samples were accepted for this study group.

Twenty-five cases of IBD as per the original histopathology report were included in this study. Since infiltration of deeper tissue layers is a major criterion to distinguish intestinal inflammation from lymphoma (Kiupel, Smedley, Pfent, Xie, Xue, Wise, DeVaul, & Maes; 2011) only full thickness biopsies were included in this study group. Of the 179 cases identified as IBD, 66 cases had surgical full thickness biopsies available. Twenty-one cases were rejected due to an inflammatory infiltrate that was not predominantly lympho-plasmacytic, additional architectural changes that were not attributable to IBD alone and likely to hinder assessment of the mucosa (e.g. autolysis, freezing artifacts, polyps, intussusceptions, edematous or ulcerated mucosa) and one case tested positive for FeLV. The twenty-five included cases were selected with due regard to getting an IBD group that equally represents the years 2007 through 2011 as well as an even distribution of mild, moderate, and severe IBD grades.

Thirty-two cases of small cell, low grade lymphoma according to the original histopathology report were included in this study. They were randomly chosen to equally represent the years 2007 through 2011. Surgical full thickness biopsies and samples from necropsies were preferred over endoscopic samples but included in the study only if the alignment and preservation of the sections allowed unhindered mucosal assessment. Likewise, two cases that had tested positive for FIV were rejected. Additional endoscopic sections for a particular year were included if enough full thickness cases were not available for the respective year, if immunohistochemistry staining had already been done, or from cases in which histopathology reports specifically mentioned that the lymphomas had likely arisen from underlying IBD. Endoscopic biopsy specimens were included only if a diagnosis of lymphoma could still be made with certainty. This required the presence of typical lymphoma features such as epitheliotropism (Kiupel et al., 2011) whose assessment is not impaired in endoscopic biopsy samples.

All sixteen large cell, high grade lymphomas identified were included in this study. Endoscopic and surgical biopsies were accepted for this study group. In cases of surgically removed intestinal masses

that had been consecutively sectioned, sections from within the mass rather than surgical margins were analyzed.

2.2 Histopathology

As previously described, initial inclusion of cases in this study was based on review of the histopathological descriptions and diagnoses in their respective pathology reports. Cases had been evaluated and reports written by various pathologists, report descriptions varied in detail and schemes used to reach the diagnosis were not clearly evident in every case. Therefore, H&E stained sections from every case included were reviewed by a board-certified pathologist (Dr. Sean P. McDonough (SPM), Section of Anatomic Pathology, Cornell University, Ithaca, NY, USA) who was blinded to the origin of the sections.

At the time of study, formalin-fixed, paraffin-embedded (FFPE) tissues were archived in the Section of Anatomic Pathology, Cornell University. When available, the original H&E slides were reviewed. If not available, a new section was stained with H&E. Each slide was assigned to the group normal to minimally changed gastrointestinal tract, the IBD group or the lymphoma group, based on the diagnostic algorithm to differentiate intestinal lymphoma from IBD described by Kiupel et al. (2011). Each slide within the normal to minimally changed and the IBD group was graded according to the World Small Animal Veterinary Association (WSAVA) scheme (Day, Bilzer, Mansell, Wilcock, Hall, Jergens, Minami, Willard, &Washabau, 2008). Features that were graded separately were then summed to yield a composite score and assign a final grade with a total score of 0-2 being considered a normal gastrointestinal tract [0], 3-7 mild IBD [1], 8-13 moderate IBD [2], and >13 severe IBD [3]. Features evaluated for cases of lymphoma included cell size, grade, location of neoplastic cell population (intravascular, serosa, muscularis, submucosa, lamina propria, epitheliotropism), crypt hyperplasia, villus atrophy, villus fusion, epithelial erosion, ulceration, epithelial degeneration (attenuation, brush border, separation, vacuolation), and lineage by use of immunohistochemistry. After 30 days, all slides were randomized and assessed a second time. Discrepant results were resolved by a third round with the final grades determined by consensus. If multiple sections were read per case, the total score for the case in each category represented the largest extent of changes seen in any of the sections.

2.3 Immunohistochemistry

Immunohistochemical (IHC) analysis was performed on all 50 cases of intestinal lymphoma according to the reviewing pathologist (SPM). The detection of CD3+ and Pax5 as markers for T- and B-lymphocytes respectively was considered sufficient to yield a lineage diagnosis and the use of anti-CD3+ and Pax5 antibodies in feline intestinal tissue has been previously described and optimized (Janeczko et al., 2008; Willmann, Müllauer, Guija de Arespacochaga, Reifinger, Mosberger, & Thalhammer, 2009). Anti-Pax5 immunostaining is routinely used in a broad range of species at Cornell, including the cat (SPM, personal communication 4/16/2012). Slides from cases on which IHC analysis had been done previously were borrowed from the Diagnostic Center for review and for the remaining cases staining was set up afresh.

IHC staining was performed on FFPE tissue using the Dakocytomation Auto Stainer *plus* according to the manufacturer's instruction (Dako Corporation, Carpinteria, CA, USA). Polyclonal rabbit anti-

CD3+ (cat#A0452, Dako Corporation, Carpinteria, CA, USA; 1: 100) and monoclonal mouse anti-Pax5 (cat#08-1411, Invitrogen Corporation, Carlsbad, CA, USA; prediluted) primary antibodies were detected with streptavidin-biotin-peroxidase (LAB- SA) detection systems (Histostain SP Bulk Kit for Rabbit polyclonal antibodies cat#956143B and Histostain SP Bulk Kit for Mouse monoclonal antibodies cat#956543B, Invitrogen Corporation, Carlsbad, CA, USA) and the chromogen 3,3-diaminobenzidine-tetra hydrochloride (DAB, Dakocytomation, Carpinteria, CA, USA). Antigen retrieval was done by digestion with pepsin for 20- 45min for CD3+ and by microwaving in EDTA pH 8.0 for 20min for Pax5. Slides were counterstained with Hematoxilin (Dakocytomation, Carpinteria, CA, USA). Negative controls were performed by substituting the primary antibody with non-specific mouse anti-cat or non-specific rabbit anti-cat antibodies respectively (Negative mouse cat #N1698 and Negative rabbit cat#N1699; Dako Corporation, Carpinteria, CA, USA).

IHC slides were read by a board-certified pathologist (SPM).

2.4 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) on FFPE specimens for the most part was conducted as previously described by Janeczko et al. (2008) and Priestnall et al. (2004). Three recuts of the archived paraffin blocks per case and biopsy site analyzed were ordered at the Diagnostic Center. Histological sections of 4µm were cut and mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were then stored in a dry and dark place until processing.

Since some of the specimens had been archived for several years prior to being processed, minor adjustments to the above mentioned FISH protocols were made to optimize deparaffinization and staining of the tissue. Slides were heat-pretreated in an incubator (20min, 60°C) and sections subsequently deparaffinized and rehydrated by passage through xylene (3x 30minutes), 100% alcohol (2x 7min), 95% ethanol (1x 7min), and finally 70% ethanol (1x 7min). The slides were allowed to air dry. Permeability of bacterial cell walls was enhanced by incubating the sections with 30µl of Lysozyme (Sigma, #L-6876, 70'000 units/ml, St. Louis, MO, USA) under a coverslip (30min, 37°C). The slides were then rinsed with ultrapure sterilized water and promptly mounted with DNA probe mix (Integrated DNA Technologies, Coralville, IA, USA) for hybridization.

For assessment of bacterial numbers and spatial distribution, the eubacterial probe EUB-338 (GCT GCC TCC CGT AGG AGT) 5'-labeled with Cy3 was applied simultaneously with the irrelevant probe non-EUB-338 (ACT CCT ACG GGA GGC AGC) 5'-labeled with 6-FAM to control for non-specific hybridization. Both DNA probes were reconstituted with sterile water and diluted to a working concentration of 5ng/µl with a hybridization buffer appropriate to the eubacterial probe (100mM Tris, 0.9 M NaCl, 0.01% SDS, 35% formamide, pH 7.5) (Amann, Binder, Olsen, Chisholm, Devereux, & Stahl, 1990). 30µl of the probe mix and a coverslip were applied to the sections and the slides were placed in a humid hybridization chamber at 46°C overnight (12-14h) for hybridization. Washing was performed in wash buffer (100mM Tris, 0.9 M NaCl, pH 7.5; 20min, 48°C) and the slides were then rinsed with sterile water. Finally, sections were mounted with ProLong® Antifade Gold (Molecular Probes, Inc., Eugene, OR, USA) and a coverslip and sealed with clear nail polish. Stained slides were covered to preserve the light sensitive probes and stored in a walk-in cooler (4°C) until reading and between reads. Probe specificity was controlled by including positive control slides prepared from cultured *Escherichia coli DH5α* and *Streptococcus bovis* in each assay.

Slides were read with an Olympus BX51 (Olympus America, Melville, NY, USA) epifluorescence microscope and images captured with an Olympus DP-7 camera. The spatial distribution of bacteria

present was determined in 40x magnification in each section and each spatial category was then assigned a semi quantitative grade (no bacteria [0], 1-3 bacteria [1], 4-6 bacteria [2], and >6 bacteria [3]) reflecting the bacterial number in the area of densest colonization. Spatial categories evaluated were bacteria in luminal debris, in epithelium associated mucus, adherent to the epithelium, invading the mucosa, adherent to the serosal surface, and within blood vessels. The distinction between the categories luminal debris and mucus was made based on the presence of DAPI positive nuclei or residual cell structures in debris but not mucus. Bacteria were counted as being adherent to the epithelium or the serosal surface if they were tightly aligned with it over their entire length. Generally, in order not to mistake probe crystals or cellular debris for bacteria, every bacterium like structure was evaluated for red, green, and blue fluorescence and only considered to be real if it clearly showed red and blue but not green light emission. Each slide was read and graded three times total at a two month interval and the final grade for every case was determined by consensus. If multiple sections were read per case, the total score for the case in each category represented the bacterial density in the area of highest colonization in any of the slides read.

2.5 Statistical analysis

The degree of intra-observer agreement was measured by the kappa statistic (Landis & Koch, 1977; SPM, personal communication 2/20/2012). In the present study assigning cases to the normal or IBD groups on the basis of histopathology was not reliable on the basis of repeated evaluation (kappa = 0.6508). Thus, it was decided to combine cases with histopathologically normal small intestines [0] and cases of only mild IBD [1] into a minimally changed group (MC) and cases of moderate IBD [2] and severe IBD [3] into a group of assured lymphoplasmacytic enteritis (LPE) for statistical purposes. Likewise, one case of large cell lymphoma, even though of low grade and one case of mixed large cell and small cell lymphoma were included in the large cell, high grade lymphoma group (LCL) for statistical purposes.

Qualitative differences in the spatial distribution of bacteria between the different study groups were compared by use of two tailed Fisher's Exact Test in MC vs. LPE vs. small cell, low grade lymphoma (SCL) vs. LCL. Results were considered significant when at $p \leq 0.05$. Differences in MC vs. LPE vs. T-cell vs. B-cell; Normal [0] vs. IBD grade [1] vs. grade [2] vs. grade [3] vs. Neut [0] vs. Neut [1] vs. Neut [2] vs. Neut [3], and SCL non- ulcerated vs. SCL ulcerated vs. LCL non- ulcerated vs. LCL ulcerated were only depicted graphically.

For analysis of bacterial numbers in the area of densest colonization at the different locations, the same semi quantitative grades were used as for reading FISH slides (no bacteria [0], 1-3 bacteria [1], 4-6 bacteria [2], and >6 bacteria [3]). One way ANOVA (Kruskal Wallis, non-parametric) was used to compare median values of the surrogate categories of the different groups at the same location. Groups were MC vs. LPE vs. SCL vs. LCL, and spatial categories were debris, mucus, adherent, invasive, serosal, and vascular. If the Kruskal Wallis test was significant, all of the groups were compared with Dunn's Test to see where the differences between groups lie. Results were considered significant when at $p \leq 0.05$.

Statistical analyses were performed using GraphPad Prism version 4.00c for Macintosh (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Characterization of study group

The expected intra-observer kappa of the reviewing pathologist is 0.85 or greater (Landis & Koch, 1977; SPM, personal communication, 2/20/2012). On the basis of two reads the observed kappa in the present study was 0.6508 (standard error 0.0813; 95% Confidence Interval lower limit 0.4915 and upper limit 0.8101), so slides were read for a third time and the final grade determined by consensus in cases of discordant results. It was determined that 50% of the discord was related to sections from normal intestinal tissue and mild enteritis where a change in any single parameter by one grade point resulted in a case shifting category (SPM, personal communication 2/20/2012). Thus, it was decided to combine cases with histopathologically normal small intestines [0] and cases of only mild IBD [1] into the MC and cases of moderate IBD [2] and severe IBD [3] into the LPE group.

Following review, the study groups were composed as follows (TABLE 1): MC (n= 18), LPE (n= 20), SCL (n= 33), LCL (n= 17), thereof fifteen large cell, high grade lymphomas, one large cell, low grade lymphoma, and one mixed large and small cell lymphoma. Three of the cases originally diagnosed as IBD moved to the SCL group after consulting H&E and IHC sections. The change in numbers in the MC and IBD groups arose as a result of combining normal small intestines and cases of mild IBD into the MC group and cases of moderate and severe IBD into the LPE group. One case originally considered to be an early lymphomatous lesion was classified as a severe case of IBD upon review. Biopsy samples from that case were harvested endoscopically and didn't meet the inclusion criteria for the IBD group. The case was excluded from the study. One case of lymphoma with an ambiguous pathology report that was selected as a presumptive SCL proved to be LCL and changed the group. Two cases included in the LCL group turned out to be a large cell, low grade lymphoma and a mixed small and large cell lymphoma.

The MC group was composed of four cases with a normal small intestine (overall grade [0], composite scores ranging from 1-2), and fourteen cases of mild enteritis (overall grade [1], composite scores ranging from 3-7). Fourteen tissue samples were taken surgically, three endoscopically, and one at necropsy. Samples originated from duodenum (n= 10), jejunum (n= 3), and small intestine not further specified (n= 6). Two anatomic sites were read in one case (duodenum and jejunum). Samples of all but two cases were sent in from private practitioners. Patients were male neutered (n= 10), female spayed (n= 7), and intact male (n= 1) Domestic Shorthair (n= 10), Domestic Longhair (n= 2), Himalayan (n= 3), Maine Coon (n= 1), Sphinx (n= 1), and mixed breed (n= 1) cats. Age ranged from 3 to 15 years (mean= 9 years, 2 months).

Within the LPE group, there were fourteen cases of moderate IBD (overall grade [2], composite scores ranging from 8-13), and six cases of severe IBD (overall grade [3], composite scores ranging from 14-22). Full thickness biopsies were available from all cases, thereof nineteen were taken surgically and one at necropsy. Sites sampled were the duodenum (n= 13), jejunum (n= 3), ileum (n= 1), and small intestine not further specified (n= 4). Of one case, two anatomic sites were read (jejunum and ileum). Four samples stemmed from patients of the Cornell University Hospital for Animals (CUHA), sixteen from referred cases. The patient group was composed of female spayed (n= 7), intact female (n= 2), and male neutered (n= 10) Domestic Shorthair (n= 15), Domestic Longhair (n= 2), Ragdoll (n=1), and Siamese (n= 1) cats. Of one shelter cat age, sex, and breed remained unknown. Age ranged from 3 to 20 years (mean= 10 years, 11 months).

Samples from the thirty-three cases of SC lymphoma were taken at surgery (n= 20), during endoscopy (n= 12), and at necropsy (n= 1). Sites sampled were the duodenum (n= 16), ileum (n= 2),

and small intestine not further specified (n= 16) and in three cases two slides of the same case were read (small intestine not further specified). Tissue samples of four cases were obtained from CUHA patients, twenty-nine were sent to the Section of Anatomic Pathology for diagnosis. Male neutered (n= 18), female spayed (n= 14), and intact female (n= 1) Domestic Shorthair (n= 27), Domestic Longhair (n= 4), Bengal (n= 1), and Maine Coon (n= 1) cats constituted this study group. The mean age was 12 years, 4 months, ranging from 3 years and 5 months to 18 years and 5 months.

Fourteen LCL specimens originated from surgically removed intestinal masses in the duodenum (n= 1), jejunum (n= 10), ileum (n= 1), and small intestine not further specified (n= 2) and a duodenal mass that was endoscopically sampled (n= 1). Surgically removed masses were consecutively sectioned and sections analyzed stemmed from within the mass rather than the surgical margins. Only one section was analyzed in every case. Five patients had surgery done at CUHA. Affected patients were male neutered (n= 10), female spayed (n= 4), and intact female (n= 1) Domestic Shorthair (n= 14) and Domestic Longhair (n= 1) cats ranging from age 8 to 17 years (mean= 13 years, one month). One sample of LCL low grade lymphoma was available. The endoscopic pinch biopsy was taken from the small intestine (not further specified) was referred by a private practitioner. The patient was an 8 year old intact female Domestic Shorthair cat. Besides, one sample of mixed LCL SCL was available. It stemmed from a 21 year old female spayed Domestic Shorthair cat that presented at CUHA with an intestinal mass of the jejunum that was surgically removed.

Table 1: Demographics of study groups

group (n)	grade (n)	lineage (n)	biopsy (n)	site (n)	age $\bar{x}$(range)	sex (n)	breed (n)
MC (18)	[0] (4) [1] (14)		S (14) E (3) N (1)	D (10) J (3) SI (6)	9y2m (3y-15y)	mn (10) fs (7) m (1)	DSH (10) DLH (2) Him (3) Maine C (1) Sphinx (1) mixed (1)
LPE (20)	[2] (14) [3] (6)		S (19) N (1)	D (13) J (3) I (1) SI (4)	10y11m (3y-20y)	mn (10) fs (7) f (2)	DSH (15) DLH (2) Rag (1) Siam (1)
SCL (33)	LG (33)	T-cell (33)	S (20) E (12) N (1)	D (16) I (2) SI (16)	12y4m (3y5m-18y5m)	mn (18) fs (14) f (1)	DSH (27) DLH (4) Bengal (1) Maine C (1)
LCL (17)	HG (15) LG (1) LG & HG (1)	T-cell (8) B-cell (7) T-&B-cell (1) none (1)	S (15) E (2)	D (2) J (11) I (1) SI (3)	13y (8y-21y)	mn (10) fs (5) f (2)	DSH (16) DLH (1)

LG: low grade; HG: high grade; S: surgical; E: endoscopical; N: taken at necropsy; D: duodenum; J: jejunum; I: ileum; SI: small intestine not further specified; mn: male neutered; fs: female spayed; m: intact male; f: intact female; DSH: Domestic Shorthair; DLH: Domestic Longhair; Him: Himalayan; Maine C: Maine Coon; Rag: Ragdoll; Siam: Siamese

3.2 Immunohistochemistry

Of the thirty-three cases of SCL, all showed low grade proliferation and were of T-cell immunophenotype.

The LCL group was considerably more diverse (FIGURE 1). Out of the sixteen mere LC lymphomas, fifteen (94%) showed a high grade proliferation and one case was of the low grade type (6%). Seven cases (44%) stained positive for Pax5 (B-cell) and eight (50%) for CD3+ (T-cell). In one case (6%) detection of both antigens was negative. The one case of mixed LCL SCL was of mixed grades and positive for B-cell as well as T-cell markers.

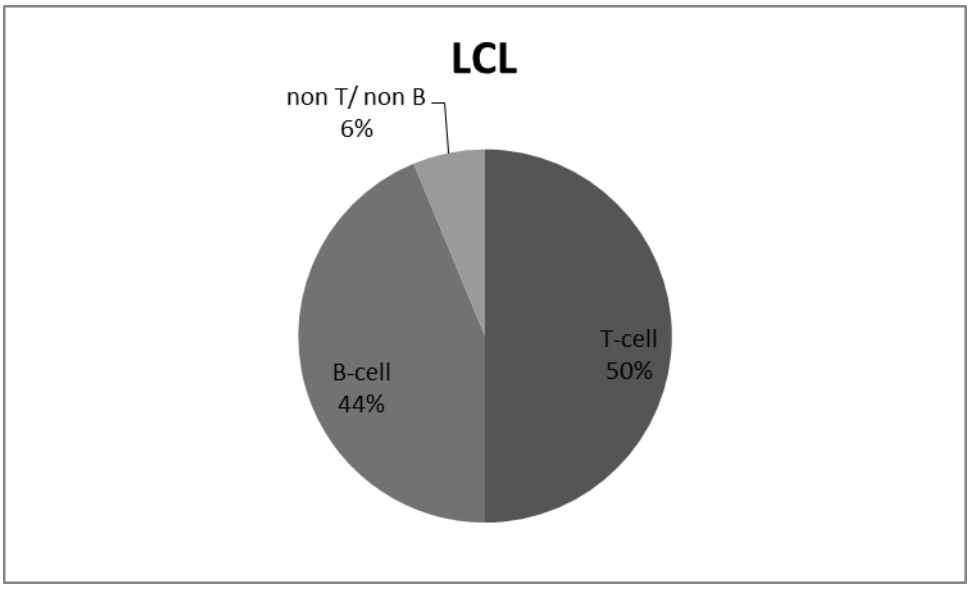

Figure 1: Distribution of immunophenotypes in large cell lymphoma (n=16).

3.3 Presence and spatial distribution of mucosal bacteria

The presence of bacteria in luminal cellular debris, invasive bacteria, and the status of blood vessels could be assessed in every case (MC n= 18, LPE n= 20, SCL n= 33, LCL n=17). In two cases of SCL and eight cases of LCL, bacteria in villus associated mucus and adherent to the epithelium could not be assessed due to mucosal ulceration (SCL n= 31, LCL n= 9). Serosa was present and could be assessed as a spatial category in most surgical samples and samples originating from necropsies (MC n= 13, LPE n= 17, SCL n= 19, and LCL n= 14). FIGURE 2 and TABLE 2 show the proportion (%) of FISH positive cases.

Bacteria were observed more frequently within luminal debris in SCL and LCL than MC (TABLE 2). LCL also had more bacteria than LPE. Bacterial presence in debris did not differ significantly between MC vs. LPE, LPE vs. SCL, and SCL vs. LCL.

Bacteria in mucus were found significantly more often in LCL than LPE. Their presence did not statistically differ between MC vs. LPE, MC vs. SCL, MC vs. LCL, LPE vs. SCL, and SCL vs. LCL. The frequency of bacteria adherent to the villus epithelium did not significantly differ between any of the groups.

Invasive bacteria were present significantly more often in LCL as compared to MC, LPE, and SCL. No statistically significant difference was evident between MC vs. LPE, MC vs. SCL, and LPE vs. SCL.

Bacteria adherent to the serosa were seen significantly more often in LCL than MC, LPE, and SCL. Their occurrence did not significantly differ between MC vs. LPE, MC vs. SCL, and LPE vs. SCL.

No bacteria were present intravascularly in MC, LPE, and SCL but in five cases of LCL. This difference was statistically significant.

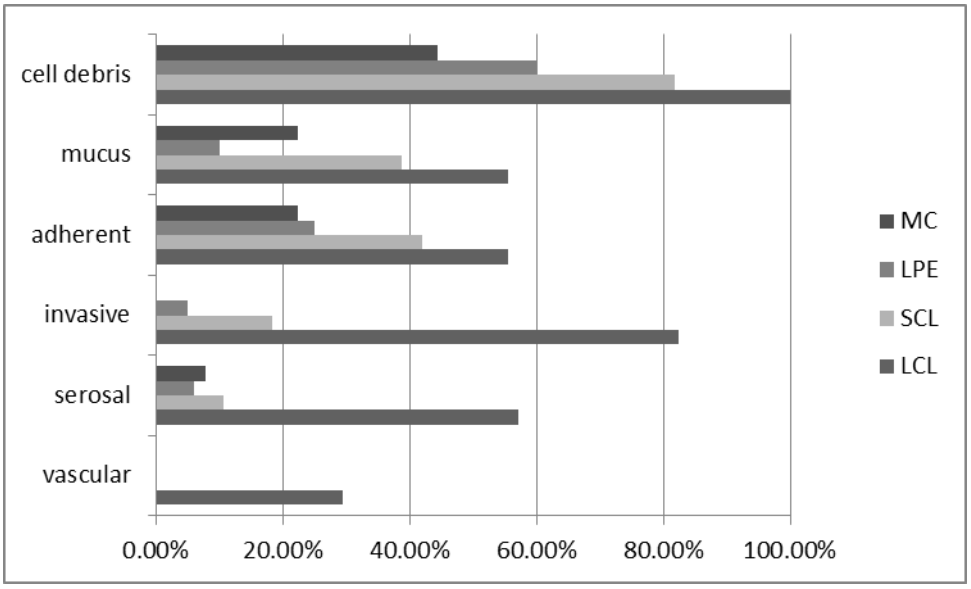

Figure 2: Spatial distribution of mucosal bacteria in different lymphoma cell morphology. The x axis shows the proportion of FISH positive cases (%).

Table 2: Differences in spatial distribution of mucosal bacteria according to lymphoma cell morphology.

	cell debris n_{pos} (%)	**mucus** n_{pos} (%)	**adherent** n_{pos} (%)	**invasive** n_{pos} (%)	**serosal** n_{pos} (%)	**vascular** n_{pos} (%)
MC	8(44)	4(22)	4(22)	0(0)	1(8)	0(0)
LPE	12(60)	2(10)	5(25)	1(5)	1(6)	0(0)
SCL	27(82)**	12(39)	13(42)	6(18)	2(11)	0(0)
LCL	17(100)$^{***,\#\#}$	5(56)$^{\#\#}$	5(56)	14(82)$^{***,\#\#\#,\wedge\wedge\wedge}$	8(57)$^{**,\#\#\#,\wedge\wedge}$	5(29)$^{*,\#\#\wedge\wedge}$

Fisher's Exact Test, vs. MC: *= p≤0.05, **= p≤ 0.01, ***= p≤0.001; vs. LPE: #= p≤0.05, ##= p≤0.01, ###= p≤0.001; vs. SCL: ^= p≤0.05, ^^= p≤0.01, ^^^p= ≤0.001.

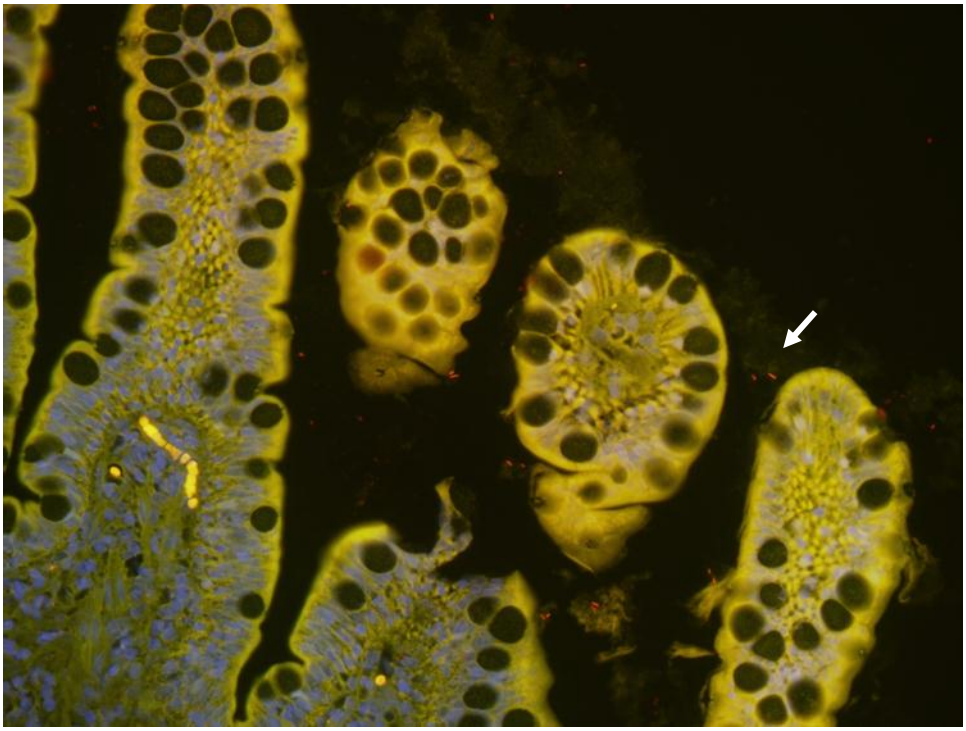

Figure 3: FISH analysis of LPE mucosa. Bacteria in villus associated mucus are red (EUB-338-Cy3), nuclei are blue (DAPI).

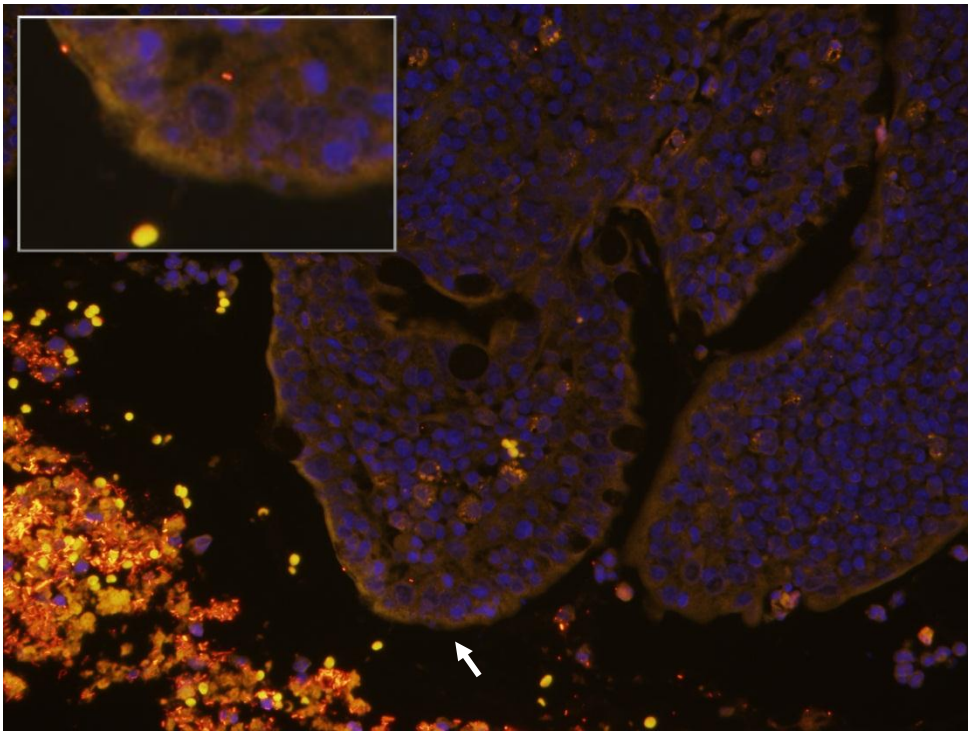

Figure 4: FISH analysis of LCL mucosa showing bacteria (EUB-338-Cy3, red) in luminal debris and bacteria invading (↗) an intact villus.

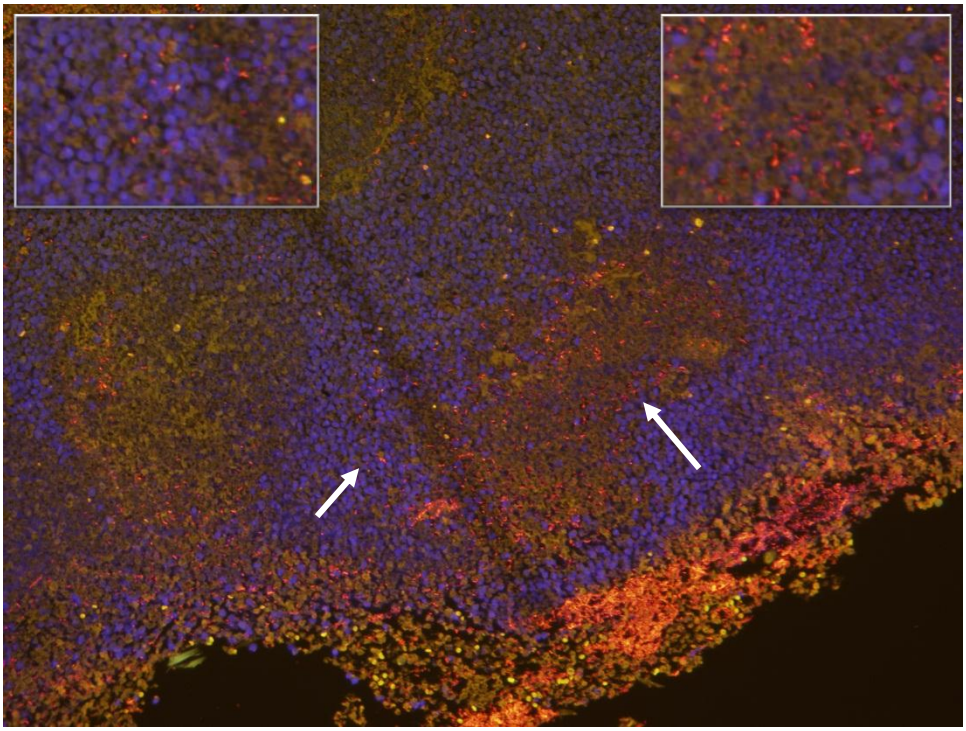

Figure 5: FISH analysis of ulcerated LCL mucosa. Bacteria (EUB-338-Cy3, red) invading necrotic foci (↖) as well as viable mucosa (↗).

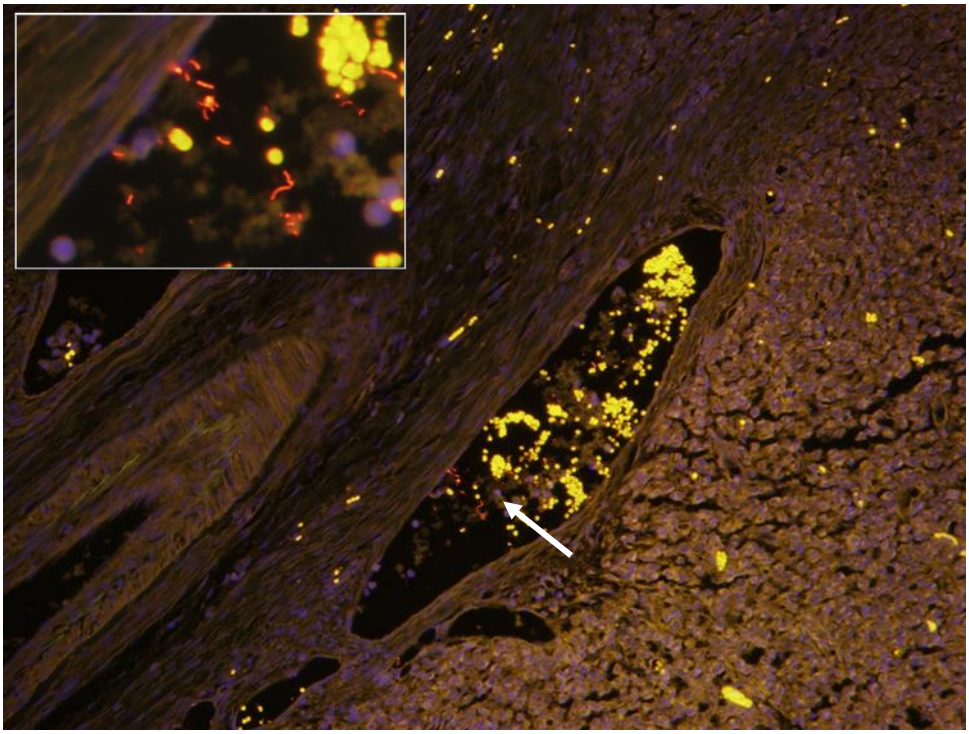

Figure 6: Intravascular bacteria in LCL (FISH).

Since the T-cell group was dominated by SC lymphomas (33 of 41, 80%), the distribution of mucosal bacteria according to lymphoma phenotype yielded similar results (FIGURE 7). The case of mixed T- and B-cell lymphoma and the non-T-, non-B-cell case were excluded from FIGURE 7 (T-cell n= 41, B-cell n= 7).

Bacteria in debris were seen more often in both types of lymphoma as compared to MC and LPE. Bacteria in mucus and adherent to the epithelium were seen more often in B-cell lymphoma than in any other group. Invasive, serosal and intravascular bacteria were seen more often in cases of T-cell and B-cell lymphoma than in MC or LPE.

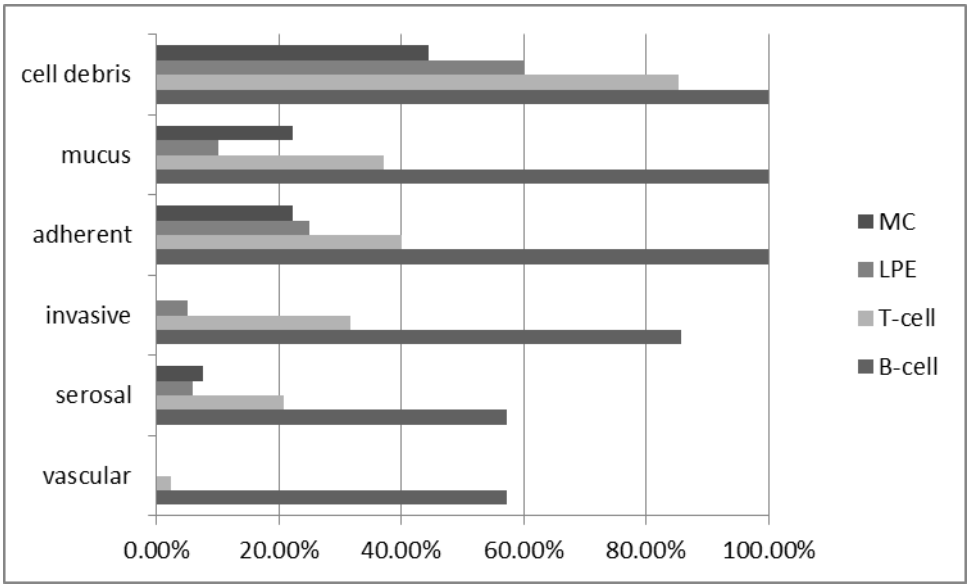

Figure 7: Spatial distribution of mucosal bacteria in different lymphoma immunophenotype. The x axis shows the proportion of FISH positive cases (%).

A higher IBD grade or neutrophilic infiltrates did not correlate with differences in the spatial distribution of mucosal bacteria (FIGURE 8 and 9). There was little difference between MC and LPE on the basis of grade of presence of neutrophils. Invasive bacteria were rarely observed and restricted to severe progressed IBD [3].

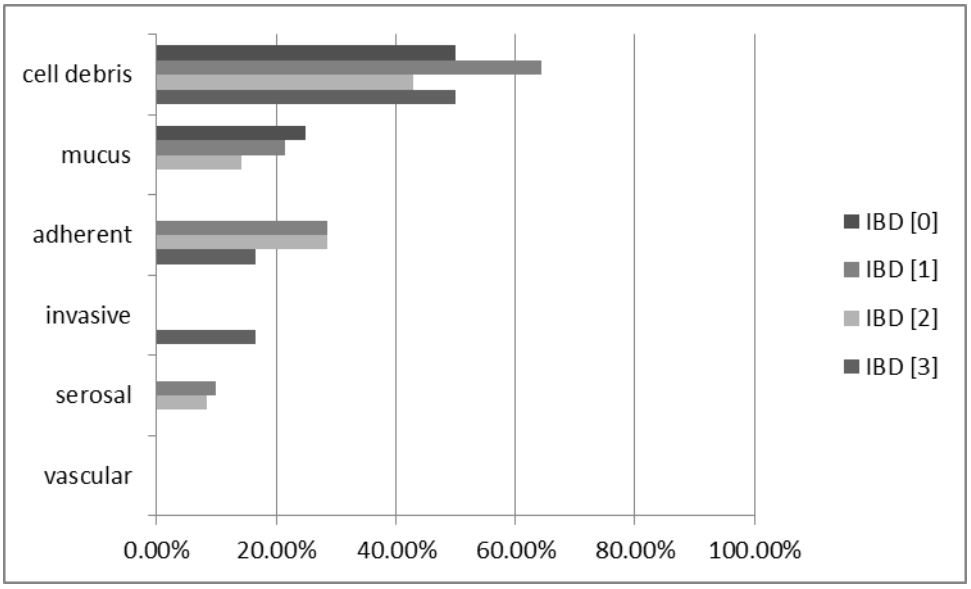

Figure 8: Spatial distribution of mucosal bacteria in different IBD grades.

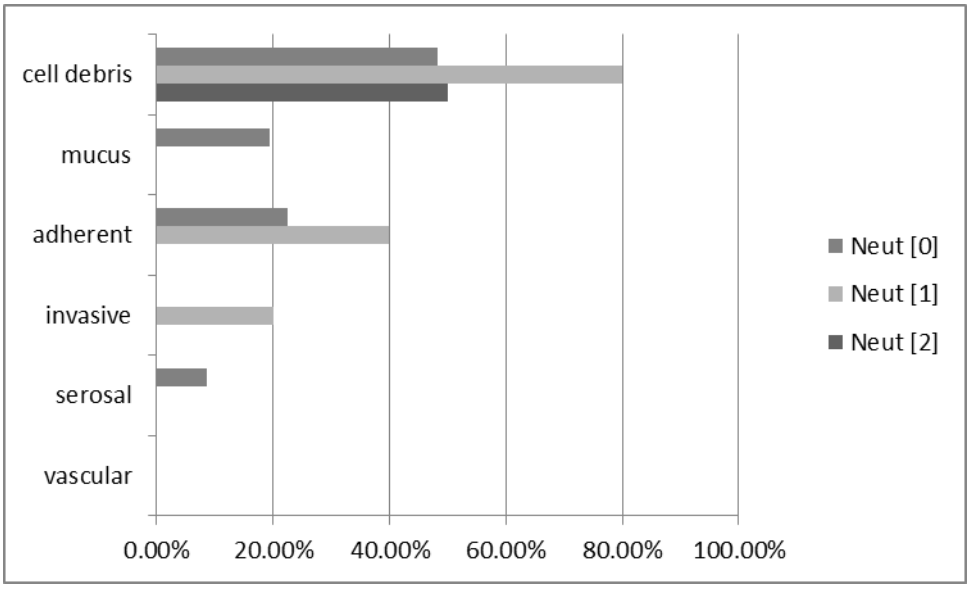

Figure 9: Spatial distribution of mucosal bacteria in relation to neutrophilic infiltrates in IBD.

Prevalence of bacteria seems to be highest in cases of ulcerated LC lymphoma in every category (SCL non-ulcerated n= 31, SCL ulcerated n= 2, LCL non-ulcerated n= 2, LCL ulcerated n= 15, FIGURE 10).

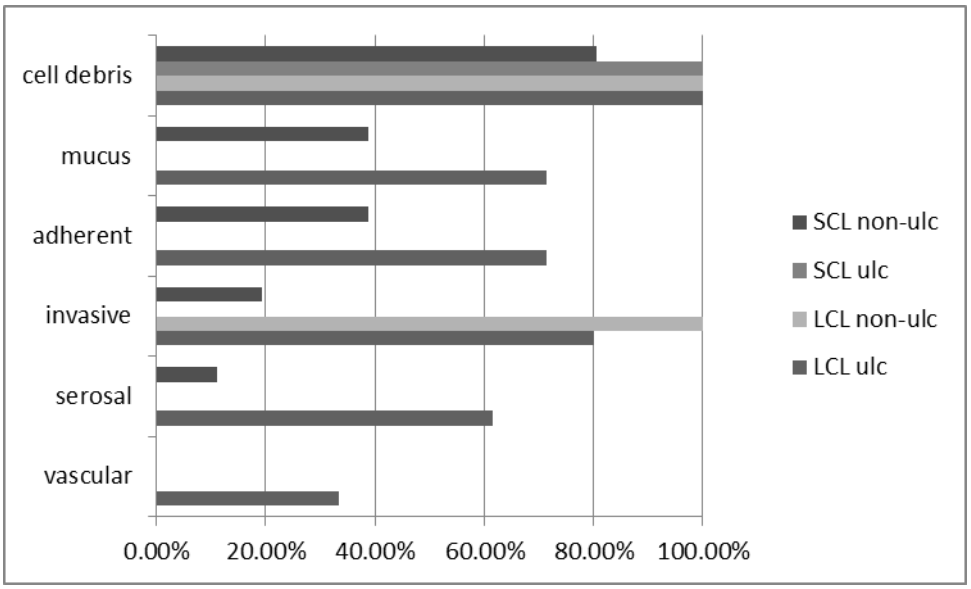

Figure 10: Spatial distribution of mucosal bacteria in ulcerated vs. non-ulcerated lymphoma.

3.4 Differences in numbers of mucosal bacteria

Bacterial numbers in luminal cellular debris, invasive bacteria, and the status of blood vessels could be assessed in all cases (MC n= 18, LPE n= 20, SCL n= 33, LCL n=17). In two cases of SCL and eight cases of LCL, bacteria in villus associated mucus and adherent to the epithelium could not be assessed due to mucosal ulceration (SCL n= 31, LCL n= 9). Serosa was present and could be assessed as a spatial category in most surgical samples and samples originating from necropsies (MC n= 13, LPE n= 17, SCL n= 19, and LCL n= 14).

The results of the quantitative analysis were very similar to what the presence/ absence comparison of the spatial distribution of mucosal bacteria revealed (TABLE 3). SC lymphoma showed significantly higher numbers of bacteria in luminal debris than MC (median 4-6 bacteria vs. no bacteria, $p<0.01$). Higher numbers of debris bacteria were additionally seen in LCL as compared to MC (median >6 bacteria vs. no bacteria, $p<0.001$), LPE (median >6 bacteria vs. 1-3 bacteria, $p<0.001$), and SCL (median >6 bacteria vs. 4-6 bacteria, $p<0.05$).

Significantly more bacteria were found in villus associated mucus of LCL than LPE (median 1-3 bacteria vs. no bacteria, $p<0.05$). The number of bacteria found to adhere to the epithelium did not significantly differ between any of the groups.

Numbers of invasive bacteria were significantly higher in LCL than in any other group (median >6 bacteria vs. no bacteria, $p<0.001$).

Adherent to the serosa, significantly more bacteria were seen in LCL than in MC, LPE (median >6 bacteria vs. no bacteria, $p<0.001$), and SCL (median >6 bacteria vs. no bacteria, $p<0.01$).

Likewise, intravascular bacteria were seen in significantly higher numbers in LCL than in any other group (range 0 to >6 bacteria vs. no bacteria in MC $p<0.01$, vs. no bacteria in LPE and SCL, $p<0.001$).

Table 3: Differences in median numbers of mucosal bacteria

	cell debris median (range)	**mucus** median (range)	**adherent** median (range)	**invasive** median (range)	**serosal** median (range)	**vascular** median (range)
MC	0(0-1)	0(0-3)	0(0-3)	0(0-0)	0(0-1)	0(0-0)
LPE	1(0-3)	0(0-3)	0(0-3)	0(0-1)	0(0-3)	0(0-0)
SCL	2(0-3)**	0(0-1)	0(0-3)	0(0-3)	0(0-3)	0(0-0)
LCL	3(1-3)$^{***,\#\#\#,\wedge}$	1(0-3)$^{\#}$	1(0-2)	2(0-3)$^{***,\#\#\#,\wedge\wedge\wedge}$	1(0-3)$^{**,\#\#,\wedge\wedge}$	0(0-3)$^{**,\#\#\#,\wedge\wedge\wedge}$

Kruskal- Wallis Test/ Dunn's Test, vs. MC: *= p≤0.05, **= p≤ 0.01, ***= p≤0.001; vs. LPE: #= p≤0.05, ##= p≤0.01, ###= p≤0.001; vs. SCL: ^= p≤0.05, ^^= p≤0.01, ^^^p= ≤0.001.

4. Discussion

Chronic bacterial driven inflammation of the stomach and intestine is linked to the development of lymphoma in people and experimental animals (Al-Saleem & Al-Mondhiry, 2005; Isaacson & Du, 2005; Lecuit et al., 2004; Mueller et al., 2005; Parsonnet & Isaacson, 2004). Alimentary lymphoma has become increasingly prevalent in cats, despite the decrease in retroviral infections previously associated with feline lymphoma (Louwerens et al., 2005). Recent studies have demonstrated an association between inflammatory bowel disease and mucosal bacteria in cats (Janeczko et al., 2008), and it seems plausible that enteric bacteria could drive chronic inflammation and potentially trigger intestinal lymphomagenesis. It is against this background that we sought to determine the presence and localization of mucosal bacteria in biopsies from cats with histologically minimally changed mucosa (MC), lympho-plasmacytic enteritis (LPE) and lymphoma of small (SCL), and large cell (LCL) phenotype.

Tumor development for a long time was thought to be due to inherited or acquired genetic mutations that cause tissue to proliferate unrestrictedly. While this might hold true for a variety of neoplastic disorders in human as well as veterinary medicine, a rethinking was needed after the detection of Epstein-Barr virus (EBV) as a causative agent of Burkitt's lymphoma in people in the late 1960's. At about the same time the pathogenesis of leukemia and lymphosarcoma in cats infected with the FeLV was discovered. Nowadays, awareness of infectious agents other than viruses as carcinogens in specific types of cancer is steadily increasing. With a decline in FeLV prevalence and typically FeLV-associated lymphoma cases and an increase in spontaneously arising alimentary lymphoma (Louwerens et al., 2005; Mahony et al., 1995; Vail, 2007), it seems important to consider triggers for lymphomagenesis other than well recognized viral infections or oncogenes. This notion is reinforced by the switch from B-cell immunophenotype to small cell type and T-cell immunophenotype in feline alimentary lymphoma.

For the purpose of this study, patients that tested positive for either FeLV or FIV were excluded. Given the retrospective nature of the study, however, the patient history was often limited to information provided on the histopathology request form submitted with the tissue samples and did not always include laboratory test results. In sixteen of the 88 cases included in this study (18%), specimens were obtained from in-house patients at the CUHA and it was possible to consult medical records in greater detail. Even then, FeLV and FIV testing was not performed in every case and negative test results were not always retrievable. We therefore cannot conclude that the results of the present study represent the conditions in a cohort of virus negative cats with certainty. Further investigations with a study group whose viral status is better defined are necessary to see whether these results remain valid in the absence of FeLV and FIV and to be able to make more definitive statements about the involvement of mucosal bacteria in lymphomagenesis.

With the drop in FeLV prevalence and the increase in non-FeLV-associated lymphoma cases came a shift in patient phenotype towards alimentary lymphoma with a small cell phenotype (Fondacaro et al., 1999; Louwerens et al., 2005; Moore, Rodriguez-Bertos, & Kass, 2011; Vail, 2007). The search of our histopathological database during the years 2007 to 2011 yielded a total of 136 SCL and 16 LCL cases confirming that SCL is much more prevalent than LCL in New York State. The median age of alimentary lymphoma patients in North America reportedly is 13 years (Fondacaro et al., 1999; Kiselow et al., 2008). A tendency for male cats to be predisposed to alimentary lymphoma has been described before the switch in lymphoma immunophenotype (Mahony et al., 1995; Zwahlen et al.,

1998). In the post-FeLV era, neither a significant sex nor breed predisposition could be confirmed for alimentary lymphoma in North America (Fondacaro et al., 1999; Louwerens et al., 2005; Richter, 2003). A signalment of male, purebred cats has been reported to be overrepresented in IBD (Dennis, Kruger, & Mullaney, 1992). In the present study, five cats out of eighteen whose small intestines were minimally changed were purebred (27.8%, Himalayan n= 3, Maine Coon n= 1, Sphinx n= 1). In the LPE group, two cats were of pure breed (10%, Ragdoll n= 1, Siamese n= 1) and within the groups of both types of lymphomas, only two cats were of breeds other than Domestic Shorthair or Domestic Longhair cats (4%, Bengal n= 1, Maine Coon n= 1). Both sexes were equally represented in the LPE (10 males, 9 females, thereof 2 intact) as well as the lymphoma groups (28 males, 22 females, thereof 3 intact). The patient ages comprised a range of 3-15 years (median 9 years, 2 months) in the MC group, 3-20 years (median 10 years, 11 months) in the LPE group, 3-18 years, 4 months (median 12 years, 4 months) in the SCL group and 8-21 years (median 13 years, 1 month) in the LCL group. Inferring from this study population, there seems to be no breed nor gender predisposition for either IBD or lymphoma. It has to be said that only the MC and LCL group represent the full spectrum of cases seen at the Section of Anatomic Pathology, whereas the cases constituting the IBD and SCL groups were selected with regard to year sampled, method of sampling, and extent of histological lesions. Due to that selection bias, it was decided not to compare demographic information between the groups statistically. However, it seems noteworthy, that the IBD as well as the SCL group both contained individuals that were diagnosed with the respective diseases at an age as early as 3 years indicating that both diseases should still be included in the differential diagnoses for gastrointestinal signs even in the young patient. Dennis et al. (1992), Jergens, Moore, Haynes, and Miles (1992), and Hart, Shaker, Patnaik, and Garvey (1994) have reported similar findings for IBD.

In clinical medicine, histological grades are commonly employed to predict patient outcomes or measure the response to therapy (Goodman, 2007). No matter how precisely defined, histopathology scores invariably contain a subjective element based on observer experience and bias so there is always a degree of observer variability. The degree of observer variability can be measured by the kappa statistic, which can range from 0 (chance agreement) to 1.0 (perfect agreement). A kappa statistic of 0.81–1.0 is almost perfect agreement, 0.61–0.8 substantial agreement, 0.41–0.6 moderate agreement, 0.21–0.4 fair agreement, and 0–0.2 slight agreement (Landis & Koch, 1977). Agreement is always greater when there are fewer categories so most schemes grade each factor as 1 (mild), 2 (moderate), or 3 (severe). In the present study assigning cases to the normal or IBD groups on the basis of histopathology was not reliable on the basis of repeated evaluation (kappa = 0.6508). As 50% of the variation was due to differences in IBD grade [1] and normal [0] we combined these groups. The WSAVA scheme provides no clear guidelines on how to determine severity on the basis of standardized numerical categorization. Simply summing categorical scores assigned to different histological features has been criticized as statistically inappropriate, but this approach has been used to generate clinically useful systems in other areas of medicine (D'Amico, Garcia-Tsao, & Pagliaro, 2006). Studies in cats with IBD indicate that certain histopathological parameters are better correlated with clinical signs than others e.g. atrophy and fusion of villi correlate with clinical signs (Janeczko et al., 2008). Thus, we advocate developing a scheme that gives greater weight to those factors that predict disease severity and/ or clinical outcome.

In a review article in 2008, Wilson indicates that low grade lymphomas are more likely to be of T-cell immunophenotype and high grade lymphomas more likely to be B-cell lymphomas. The

references Gabor, Malik, and Canfield (1998) and Richter (2003) however state that to date, it has not been proven that the histological phenotype can predict lineage diagnosis in lymphoma. In our study group a small cell phenotype consistently correlated with a low proliferation rate (100%) and T-cell phenotype (100%). However, a large cell phenotype did relate to grade of proliferation, but did not reliably predict the immunophenotype with seven cases (44%) stained positive for Pax5 (B-cell) and eight (50%) for CD3+ (T-cell).

The single case of large cell low proliferation rate was positive for CD3+ suggesting that the low lymphoma grade rather than the large cell size might be predictive of immunophenotype in this type of lymphoma. Thus the positive staining for CD3+ and Pax5 in one case of mixed large cell and small cell and mixed high grade and low grade lymphoma was to be expected. A selection bias in the SCL group could be responsible for overlooked cases of small cell high grade or small cell B-cell lymphomas yet the absence of excessive selection for inclusion in the LCL group suggests that LC lymphoma truly constitutes a diverse group.

Mucosal bacterial colonization determined by FISH analysis yielded similar results when bacterial association was evaluated by the presence or absence of bacteria, or enumeration of bacteria. We found that bacteria were more frequently present and more abundant in luminal debris in LCL and SCL. Only LCL had higher numbers of bacteria in the mucus, invasive, serosal, and vascular compartments. The distribution and density of mucosal bacteria recognized by the EUB-338 probe was similar in MC and LPE and confirms the results of a previous study in cats with gastrointestinal disease by Janeczko et al. (2008). We did not evaluate the relative contributions of particular bacterial species and are therefore not able to comment on changes in the composition of the mucosa-associated microflora in LPE, SCL, and LCL. Previous studies in feline IBD indicate an increase in Enterobacteriaceae (Janeczko et al., 2008).

The absence of invasive bacteria in SCL does not preclude their involvement in tumorigeneis. The examples of *Helicobacter* colonization and its association with gastric lymphoma in people show that invasion of the mucosa is not necessary for bacteria to trigger carcinogenesis but that surface antigen receptors play an important role in the pathogenesis of lymphoproliferative lesions (Isaacson & Du, 2005). Broad range sequence based analysis of biopsies from cats with LPE, SCL, and LCL to determine the bacterial species colonizing the intestinal mucosa would help to determine if distinct bacterial signatures are associated with alimentary lymphoma. FISH analysis with probes against bacteria identified by sequencing could then be performed to determine the spatial distribution of specific bacteria.

Our finding that LCL has higher numbers of bacteria in the mucus, invasive, serosal, and vascular compartments has not been previously reported. It may be explained by the propensity for this form of alimentary lymphoma to form solid intestinal masses with effacement of normal tissue, erosion, and ulceration. It is directly relevant to acute abdomen and septicemia reported in this group, explained by the more extension of erosion and ulceration and architectural distortion associated with this group's masses (Richter, 2003; Vail, 2007; Wilson, 2008). In contrast LPE and SCL clinically present very similarly with chronic signs of vomiting, diarrhea, varying appetite, weight loss, and normal or diffusely thickened bowel loops (Dennis et al., 1992; Hart et al., 1994; Jergens et al., 1992; Richter, 2003; Vail, 2007; Wilson, 2008). The potential role of adherent bacteria and their signaling pathways in promoting LC lymphoma cannot be assessed in the present study since 47% of all LC lymphomas showed severe mucosal ulceration and an intact epithelium was not present for evaluation. The highest density of bacterial colonization in any compartment and especially the presence of invasive bacteria was associated with cases of ulcerated LC lymphoma. Unfortunately,

case numbers for non-ulcerated LC lymphomas and ulcerated SC lymphomas are too few to allow meaningful statistical evaluation of the impact ulceration has on bacterial distribution and density. To this point, it remains unclear, whether invasive bacteria are a causative factor in LC lymphomagenesis or whether they find the ulcerated mucosa typically seen in LCL permissive for colonization.

Comparison of the effect of immunophenotype on bacterial colonization in lymphoma was hampered by segregation on the basis of cell morphology i.e. the T-cell group was dominated by SC lymphomas (33 of 41, 80%). An unexpected finding of the present study was the similar prevalence of B- and T-cell immunophenotypes in high grade LCL. It would be more interesting to ask, whether there is a difference within the LCL group based on whether they are LCL T-cell or LCL B-cell. However the present study was under powered in its ability to address this question. In particular, it would be interesting to determine if bacterial colonization in feline alimentary lymphoma is more often associated with B-cell neoplasms, as seen in human syndromes for which bacterial involvement in pathogenesis has been be proven.

In conclusion, the results of the present study reveal that alimentary lymphoma is associated with alterations in the spatial distribution of mucosa-associated bacteria. Bacterial invasion is most frequent in cases of LC lymphoma, with bacterial translocation in approximately one third of cases. The high prevalence of bacterial translocation observed in LCL highlights the clinical importance of evaluating and closely monitoring cats with alimentary lymphoma for evidence of septicemia.

This study confirmed that small cell low grade lymphoma correlates with a T-cell immunophenotype. An unexpected finding was the similar prevalence of B- and T-cell immunophenotypes in high grade LCL.

A role for mucosal bacteria in the development of lymphoma needs to be determined. Future studies should be directed at defining the microbial and chemical microenvironment associated with the intestinal mucosa of healthy cats, cats with LPE, and cats with alimentary lymphoma. Our studies highlight the need to examine the relationship of immunophenotype in LCL to disease progression, treatment response, and mucosal microenvironment.

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